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Phosphorylation-induced activation of tilapia nonspecific cytotoxic cells by serum cytokines

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ABSTRACT: Cytokines as soluble mediators of immunity are important in understanding immunological mechanisms against infectious organisms and during stress conditions. In the present study, the role of protein tyrosine phosphorylation is assessed in the activation of nonspecific cytotoxic cells (NCC) from tilapia *Oreochromis niloticus* by cytokine-like serum factors. NCC are the teleost equivalent of mammalian natural killer (NK) cells. In teleost fish, NCC are important mediators of innate immunity against bacterial and parasite insult and tumor growth. We have previously shown that exposure of tilapia (a tropical fish) to cold water temperatures (3 to 5 min at 5 to 10°C) produces physiological stress responses characterized by immediate phenotypic and immunological changes. The serum obtained from stressed tilapia contains a 'stress activating serum factor' (SASF) which passively increases *in vitro* naïve NCC cytotoxicity 2- to 4-fold over control levels. In an effort to identify the mechanisms of activation of cytotoxicity by SASF, the phosphorylation status of tyrosine residues in proteins from treated NCC was determined. NCC were incubated with heat-inactivated or untreated stress serum and Western blots of the cell lysates were probed with anti-phosphotyrosine monoclonal antibodies (mabs). The levels of tyrosine phosphorylation in several proteins of the SASF-activated NCC were higher than in control cells. Increased tyrosine phosphorylation was also induced by incubation of NCC in the presence of the tyrosine phosphatase inhibitor Na orthovanadate (vanadate). In every case, an increase in phosphorylation status shown by Western blotting was correlated with increases in cytotoxic activity of NCC against HL-60 target cells. The enzyme inhibitor Herbimycin A (HA) has been previously used to inhibit the activity of the *src*-family of tyrosine kinases. In the present study, a 4 h pretreatment of NCC with HA (2 µM), followed by treatment with SASF blocked the activation of cytotoxicity produced by SASF. These results suggested that activation of NCC by cytokine-like factors is mediated through activation of the *src* family of protein tyrosine kinases. Activation was associated with increased phosphorylation and higher cytotoxic effector functions.

KEY WORDS: NCC · Tilapia · Cytokines · Phosphorylation

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INTRODUCTION

An important mechanism of innate immunity in tilapia *Oreochromis niloticus* is mediated by nonspecific cytotoxic cells (NCC), thought to be the teleost equivalent of mammalian natural killer (NK) cells (Jaso-Friedmann & Evans 1999). NCC participate actively as the first barrier of defense in stress related responses during adverse environmental conditions and following bacterial, viral or parasite infections (Jaso-Friedmann & Evans 1999, Evans et al. 2000, Jaso-Friedmann et al. 2000). We have previously demonstrated that serum from temperature-stressed tilapia contains cytotoxicity activation factor(s) (i.e. stress activated serum factors, SASF) that amplify *in vitro* NCC activity isolated from peripheral blood of naive (non-stressed) fish (Jaso-Friedmann et al. 2000). Recognition of certain effector characteristics of SASF suggests the

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presence of cytokine-like soluble mediators in the serum of tilapia that are released as one of the immediate immunological responses to stress conditions.

Cytokines constitute extracellular signaling proteins or peptides that act as local mediators in cell-cell communication (Liles & Van Voorhis 1995). Many of the cell-surface receptor proteins associated with these extracellular factors are activated by non-receptor protein tyrosine kinases (PTKs). These kinases phosphorylate various intracellular target proteins on tyrosine residues when the receptor binds its ligand (Miyahima et al. 1992, Mustelin & Burn 1993, Schreurs et al. 1993). The kinases involved with these tyrosine-kinase-associated receptors may belong to the *Src* family of non-receptor PTKs. Proto-oncogenic kinases were first described as transforming proteins in Rous sarcoma virus transformed cells. At least 8 members of the *src* family of nonreceptor PTKs have been identified in mammals: *src*, *lyn*, *yes*, *fgr*, *fyn*, *lck*, *hck* and *blk* (Mustelin & Burn 1993). Another family of non-receptor PTKs which constitutes an important component in cytokine activation of effector cells is the Janus family of enzymes (JAK1, JAK2 and Tyk2) (Argetsinger et al. 1993).

When the cytokine receptor binds its ligand on the surface of the cell, the tyrosine residues of the tyrosine-kinase-associated receptors are phosphorylated by PTKs. This phosphorylation constitutes the first step of several downstream signaling events responsible for the cell activation. Various enzyme inhibitors of tyrosine kinases have been reported as useful tools for understanding the role of a specific family in signal transduction molecules involved in cell growth, differentiation or activation. The main function that this type of inhibitors has on NK cells and T cells is related to the inhibition of the cytotoxic activity associated with cellular phosphorylation events. Herbimycin A (HA) is one of the most common inhibitors used for the *src* family of kinases (Uehara et al. 1985, Uehara & Fukazawa 1991, Li et al. 1995, Kalb et al. 1997, Sweeney et al. 1998).

As some tyrosine kinases are involved in cell activation, this could in turn be rapidly turned off by the opposite effect of a group of enzymes with tyrosine phosphatase activity. These tyrosine phosphatases induce dephosphorylation of the same type of receptor proteins (Walton & Dixon 1993). Phosphatase inhibitors (zinc chloride, okadaic acid, etc.) have been previously used to demonstrate the role of phosphoproteins in signaling responses (Fisher et al. 1991). Sodium fluoride and sodium orthovanadate appear to have preferential binding specificities for serine and tyrosine residues, respectively (Alexander 1990, Hunke et al. 1995). In addition to being a broad-spectrum potent inhibitor of protein tyrosine phosphatases, sodium orthovanadate also inhibits Na+/K+ ATPase, acid alkaline phosphatases, phosphofructokinase, and adenylate kinase (Hunke et al. 1995). Vanadate treatment has previously been shown to cause activation of catfish NCC cytotoxicity by prolonging the phosphorylation status of key tyrosine residues (Evans & Jaso-Friedmann 1994).

In the present study we determined that tyrosine phosphorylation and dephosphorylation of proteins in tilapia NCC control the activation of effector functions by cytokine-like serum factors. Activation of NCC by SASF required an increase in protein tyrosine phosphorylation. The importance of the phosphorylation status on cytotoxicity was supported by data that showed that phosphatase inhibition (by treatment with sodium orthovanadate, Na_3VO_4) maintained high levels of tyrosine phosphorylation concomitantly with heightened cytotoxic activity. The involvement of the *src* family of protein kinases in tyrosine phosphorylation was demonstrated by the inability of SASF to activate cytotoxicity in the presence of HA. Further, we also show that both *lck* and *fyn* are expressed in tilapia NCC.

MATERIALS AND METHODS

Animals. *Oreochromis niloticus* (*Tilapia nilotica*) of both sexes were obtained from AmeriCulture (Animas, NM, USA). For 30 d or more before use, tilapia were maintained in flow-through 300 gallon (1100 l) fiberglass aquaria at a controlled water temperature (25°C). Water quality monitoring included nitrite, ammonia nitrogen and chlorine determinations. The diet consisted of pelleted fish feed (Mazuri Choi, Purina Foods, St. Louis, MO, USA). Experimental fish were 85 to 100 g.

Purification of NCC. Fish were bled from the caudal vein into heparinized syringes (1000 units ml^{-1}). Cells were isolated from peripheral blood by standard techniques. Red cells were removed by 2 cycles of differential centrifugation at 650 × *g* for 20 min on Histopaque 1077 (Sigma Chemicals, St. Louis, MO, USA). Cells remaining at the gradient interface cushion were collected, washed once and resuspended in complete RPMI 1640 (Cellgro, Media Tech, Washington, DC, USA). RPMI was adjusted to 280 mOsM with H_2O and supplemented with L-glutamine, sodium pyruvate, MEM vitamin solution, MEM amino acid solution, MEM nonessential amino acid solution (Cellgro, Media Tech) 50 mg ml^{-1} gentamicin (Schering-Plough Animal Health Corp., Kenilwork, NJ, USA) and 10% heat inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA, USA).

Mab 5C6 or IgM isotype control was isolated from cell culture supernatants by ammonium sulfate precip-

itation (50% saturation) using standard techniques (Evans et al. 1988). This mab has specificity for a 32 kDa membrane protein on NCC that functions as an antigen recognition and signalling molecule in catfish (Jaso-Friedmann et al. 1997), and tilapia (Jaso-Friedmann & Evans 1999, Evans et al. 2000, Jaso-Friedmann et al. 2000). Purified NCC were 80 to 90% positive for mab 5C6 staining by flow cytometric analysis.

Stress activation. Stress-activated serum factors were generated as described (Jaso-Friedmann et al. 2000). Briefly, tilapia were immersed in cold water (5 to 15°C, for 3 to 5 min). Fish were immediately bled by caudal venipuncture and serum harvested using standard techniques. Collected sera were allowed to clot overnight at 4°C in the dark and harvested by centrifugation ($1000 \times g$ for 30 min).

Flow cytometry. Flow cytometry analysis was done in an EPICS XL-MCL 4 color SYSTEM IIR automated cell analysis system (Coulter Electronics Corp, Hialeah, FL, USA). Monoclonal antibody 5C6 was used to stain 2×10^5 cells prepared from peripheral blood (60 min/4°C). Cells were washed with cold PAB (4°C; PBS/0.1% azide/1.0% bovine serum albumin). FITC-conjugated anti-mouse IgM (50 µl of a 1:20 dilution; Sigma Chemicals) was added to each sample (30 min/4°C). Samples were washed twice and resuspended in cold PAB for analysis.

Cytotoxicity. Target cells for the chromium release cytotoxicity assays were human transformed HL-60 cells (ATCC CCL 240; human promyelocytic leukemia). Cell cultures were maintained in RPMI-1640 (Cellgro, Media Tech) containing 10% FBS. Target cells (2×10^6) were labeled with chromium-51 for 3 h at 37°C, washed 3× with RPMI and resuspended at 1×10^5 cells ml^{-1}. Equal volumes (100 µl) of labeled target cells and NCC were co-cultivated in 96-well round bottom microtiter plates for 16 h (28°C); supernatants were harvested and assayed for chromium-51 release. Percent of Specific Release (PSR) was calculated as follows: (test cpm-spontaneous cpm/total cpm-spontaneous cpm) × 100. Values expressed are means of triplicate samples.

Cell cycle analysis and determination of DNA hypoploidy. Camptothecin (CAM) (1 mM) (Sigma) was used to induce apoptosis and DNA hypoploidy *in vitro*. CAM, a DNA topoisomerase inhibitor (prevents DNA hybridization), is highly toxic to NCC cells in the S phase. Cell cycle analysis of purified NCC was performed to determine percentages of DNA hypoploidy. After different treatments and incubation times, 5×10^6 NCC were treated with 1× NIM (Nuclear Isolation Medium) for 10 min at 4°C in the dark. NIM was prepared as follows: 0.5% PI, 0.01% Triton X-100 (Sigma) and 0.1% RNAse (Sigma) in PBS pH 7.3. The DNA content was analyzed by red flourescence. The percentage of NCC hypoploidy was determined by back-gating from PI fluorescence histograms into FL2 plots.

'In vitro' cell activation and Western blot analysis. Single cells suspensions of 5×10^7 tilapia NCC obtained from PBL were treated for 20 min with heat-inactivated SASF and with SASF. Cells were washed twice with cold PBS and then lysed for 30 min in lysis buffer (10 mM Tris HCl, 0.15 M NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X100) freshly supplemented with 1 mM Na_3VO_4, 1 mM PMSF, 10 µg ml^{-1} aprotinin, leupeptin and pepstatin. After centrifugation at 4°C for 15 min, supernatants were collected as cytoplasmic extracts. Samples were boiled for 5 min in loading buffer, separated in a 11.5% acrylamide gel and transferred to nitrocellulose membranes (100 V for 60 min at 4°C). Transfer efficiency was confirmed by total protein Ponceau S staining. All incubations from this point on were done with shaking at room temperature. Membranes were blocked for 1 h in TBST containing 5% non-fat milk (Jaso-Friedmann et al. 2000) and probed with anti-phosphotyrosine monoclonal antibodies (PY-99, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were washed 4 times (5 min each) with TBST/Tween before addition of the anti-mouse IgG conjugate and detection by chemiluminescence.

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was used as a combination of 2 high-resolution electrophoretic procedures. In the first dimension, solubilized samples were separated according to their isoelectric point (pI) in gels loaded in individual glass tubes. The tube gels were prepared with the following solution: 8.25 g urea, 6 ml water, 2.0 ml of 30% acrylamide/1.8% bisacrylamide, 0.75 ml ampholytes (pH 4 to 8) and 0.3 ml NP-40. 10 µl TEMED and 70 µl of ammonium sulfate were added immediately before pouring to induce polymerizarion. The gels were pre-run for 15 min (200 V), 15 min (300 V) and 30 min (400 V) in anode (phosphoric acid 86%) and cathode (sodium hydroxide 50%) solutions. Samples were prepared (by 1:2 dilution in sample buffer: 2.85 g urea, 5 ml water, 2-mercaptoethanol and 0.4 ml ampholytes, pH 4 to 8), loaded on the gels and run for 16.5 h at 700 V. The tubes were frozen, the gels were separated and then applied to the top of an 11.5% acrylamide gel to be run at 626 V. Gels were then transferred into nitrocellulose membranes at 30 V for 12 h min at 4°C. Membranes were probed with anti-phosphotyrosine monoclonal antibodies (PY-99, Santa Cruz Biotechnology) as described above.

Kinase and phosphatase inhibitors. NCC (5×10^7) received a 4 h pretreatment with 0.5, 1, and 2 µM of HA (Calbiochem, La Jolla, CA, USA), followed by treatment with SASF. Cells were then lysed and tested by Western blot analysis as previously described. Na_3VO_4 was used as tyrosine phosphatase inhibitor. Single NCC suspensions of 50, 25, and 12.5 mM Na_3VO_4 were

prepared to perform the cytotoxicity assays. At the same time, Western blotting was performed from cell lysates of 5×10^7 NCC that were treated for 1 min with 5 mM Na_3VO_4.

RESULTS

SASF-activated NCC showed an increase in the tyrosine phosphorylation level of several proteins

It was previously reported that NCC cytotoxicity is activated by SASF (Jaso-Friedmann et al. 2000). In order to study the mechanisms of activation by SASF, the phosphorylation status of tyrosine residues in NCC cellular proteins was measured. Lysates of activated and resting NCC were separated on 11.5% acrylamide gels, followed by Western blotting with an anti-tyrosine antibody. Results showed higher level of phosphorylation in cells treated with SASF compared to control cells (data not shown). The high degree of phosphorylation in activated NCC made it necessary to run samples on 2-dimensional (2-D) gels, in order to obtain better separation of individual protein species. As shown in Fig. 1, immunoblot analysis of proteins resolved by 2-D gel electrophoresis (11.5% acrylamide) indicated a marked increase in the level of phosphorylation in several proteins of cell lysates that were treated with SASF compared to cells treated with heat-inactivated SASF.

The tyrosine phosphatase inhibitor sodium orthovanadate reduces tyrosine dephosphorylation while increasing cytotoxicity in NCC

It has been previously shown that catfish NCC cytotoxicity is increased in the presence of phosphatase inhibitors (Evans & Jaso-Friedmann 1994). Similarly, the results shown now demonstrated the importance of tyrosine phosphorylation in cytotoxicity activation by serum cytokines. In order to gain a better understanding of the factors required for cytotoxic activation, experiments were next conducted to look at the effects of prolonged tyrosine phosphorylation on the killing capacity of NCC. Purified effector cells were incubated in the presence of different concentrations of a phosphatase inhibitor (sodium orthovanadate). NCC were subsequently washed and used in cytotoxicity assays against HL-60 target cells (Fig. 2). Results showed higher cytotoxic activity with increasing concentrations of vanadate (Fig. 2a). This dose response peaked at a concentration of 25 mM Na_3VO_4. The vanadate treatment on NCC also produced an increase in tyrosine phosphorylation status (shown in Fig. 2b) which paralleled the cytotoxicity activation (Fig. 2). The concentration of Na_3VO_4 required to induce higher detectable levels of phosphorylation in activated NCC by Western blots was lower (5 mM) than the concentration required to activate for maximum cytotoxic activity (Fig. 2a). This was probably attributable to a more sensitive detection system.

Na_3VO_4 protected NCC from camptothecin-induced apoptosis

Experiments were designed to identify a possible mechanism for the increase of cytotoxic activity by Na_3VO_4 in NCC. We had previously shown (Bishop et al. 2000) that incubation of NCC in SASF augments cytotoxicity by inhibiting the activation-induced program cell death occurring following contact with target cells. This protection from apoptosis produced by SASF causes in turn an increase in the NCC's recycling

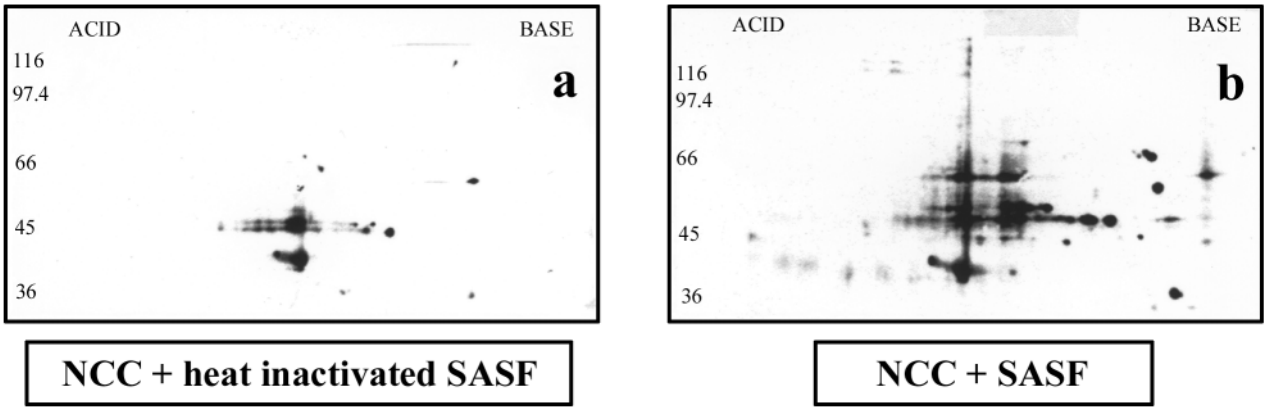

Fig. 1. Anti-phosphotyrosine Western blots from 2-D gels of NCC lysates. NCC from naïve tilapia were pre-treated with (a) heat inactivated SASF (65°C/15 min) and with (b) SASF. Cells were washed and lysates were loaded on 2-D gels. Western blots were of cell lysates were probed with anti-phosphotyrosine monoclonal antibodies (mabs)

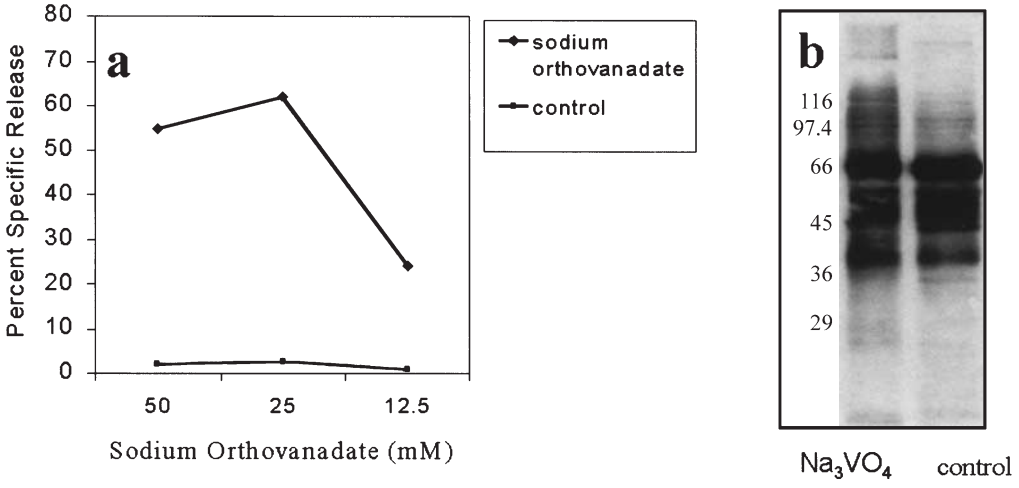

Fig. 2. Activation of NCC by sodium orthovanadate. Purified NCC from the peripheral blood of naïve tilapia were incubated with and without different concentrations of the tyrosine phosphatase inhibitor sodium orthovanadate (Na_3VO_4). Cells were added to labeled HL-60 cells (E:T ratio 160:1). (a) Supernatants were harvested and radioactivity measured following 18 h co-cultivation. (b) At the same time, using the same groups of cells, Western blots from 1-D gels of cell lysates were probed with anti-phosphotyrosine monoclonal antibodies

capacity, leading to higher cytotoxicity. To study the possibility that this was the mechanism of activation by vanadate, apoptosis of NCC was induced by incubation with 1 mM camptothecin. Fig. 3 shows a comparison of the cell cycle analysis of NCC treated with camptothecin (top, left panel) or in media alone (Fig. 3d). The diploid DNA peak is shown in Gate E, and any DNA to the left of that peak is indicative of smaller DNA fragments, i.e. hypoploidy (Gate H; Gate G is the sum of E and H). NCC treated with camptothecin showed an increase in breakdown of diploid DNA (Gate H, Fig. 3a). This breakage is characteristic of camptothecin treatment due to the onset of apoptosis. This does not occur in untreated NCC (media-treated cells, Gate H, Fig. 3c). Camptothecin could not induce apoptosis in cells that had been previously exposed to SASF (Fig. 3b) or vanadate (top, right panel). Those histograms show that pretreatment of NCC with cytokines or vanadate protected the cells from the apoptotic effects of camptothecin treatment. There was little or no DNA present in the H gate in either of the 2 histograms, indicating similar results to those of control cells.

Pretreatment of NCC with the tyrosine phosphorylation inhibitor HA reduces the activation of NCC by SASF

The increase in tyrosine phosphorylation in NCC that leads to augmentation of cytotoxicity and protection from apoptosis could be due to activation of diverse families of protein kinases. In an effort to determine the involvement of the *src* family of non-receptor tyrosine kinases, the activation of NCC by SASF in the presence of the PTK inhibitor HA was studied. Purified NCC were treated with HA followed by treatment with SASF. In Fig. 4, treatment of NCC with 1 to 2 µM HA reduced the level of killing of target cells back to control levels (NCC treated with heat inactivated cytokines). The results also correlated with a decrease in the phosphorylation levels in the anti-phosphotyrosine Western blots from 1-D gels of NCC lysates (Fig. 4b). A low concentration of HA (0.5 µM) had little effect on the phosphorylation status or on the cytotoxic activity of NCC. At higher HA concentrations phosphorylation, as well as cytotoxicity, were both inhibited. These results suggest that the increase in the killing capacity of NCC following treatment with serun cytokines required the activation of the *src* family of protein kinases.

Activated and resting NCC constitutively express *lck* and *fyn*

It is not known whether tilapia NCC express *src*-family of protein tyrosine kinases. To further investigate if these enzymes could be the targets of the HA inhibition, single suspensions of 5×10^7 NCC were treated for 20 min with SASF. Western blots of the resting or activated cell lysates were performed using policlonal antibodies against human *lck* and *fyn*. These antibodies were chosen because of their wide species crossreactivity as marketed by the manufacterer. Both *lck* (Fig. 5) and *fyn* (data not shown) were constitutively expressed in cell lysates obtained from control and SASF-treated NCC. Furthermore, phosphotyro-

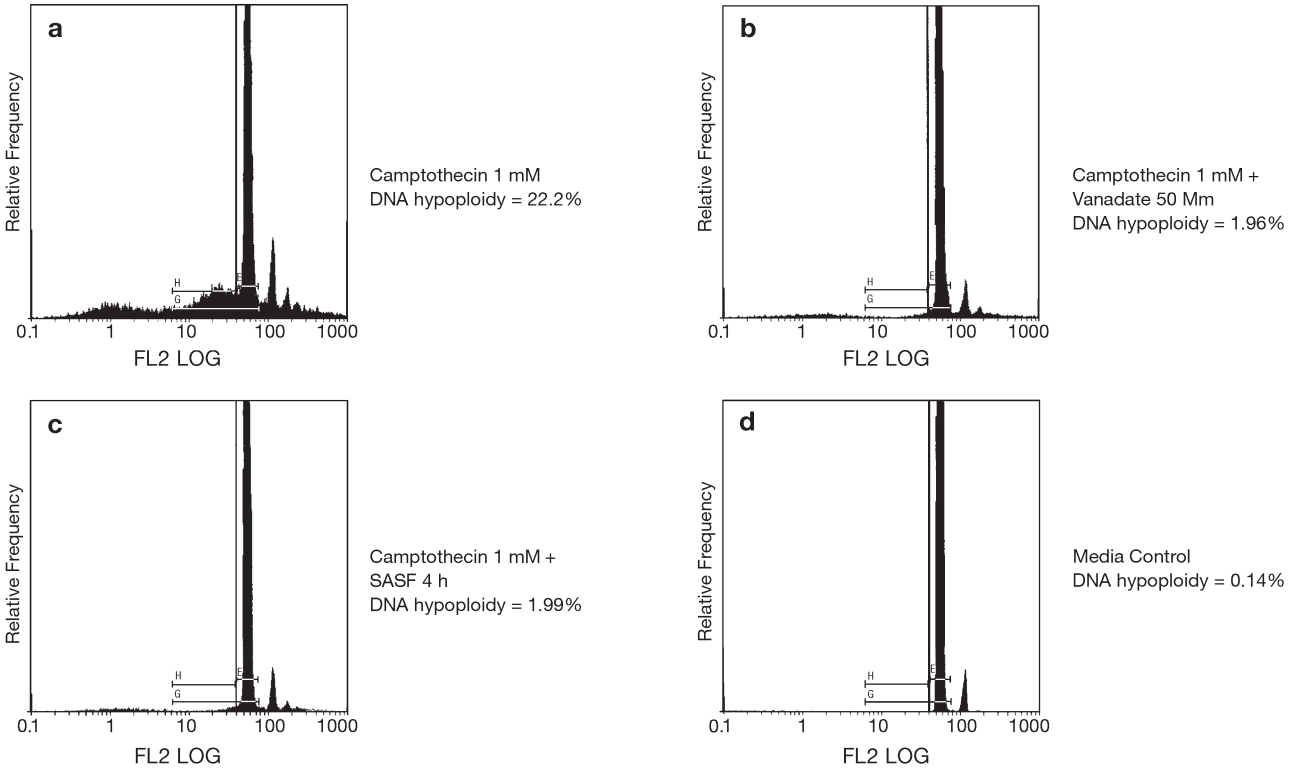

Fig. 3. Protective effects of increased phosphorylation on apoptosis of NCC. Purified NCC (10^6) isolated from peripheral blood of naïve tilapia were pre-incubated for 4 h with SASF and with sodium orthovanadate. Pretreated NCC were then incubated with camptothecin (1 mM) for 12 h (b and c). (a) Positive control. NCC treated with camptothecin. (b) Sodium orthovanadate-treated NCC were incubated in the presence of Camptothecin. (c) SASF-treated NCC were incubated in the presence of camptothecin. Gate E includes 2N (diploid DNA) and Gate H is the DNA that is smaller than 2N (denoting hypoploidy). (d) Media control

sine Western blots of the kinases showed that activation did not increase the net phophorylation status of these enzymes (data not shown). These results are in agreement with published reports of the activation of the mammalian family of *src* tyrosine kinases. Furthermore, the molecular weight of the teleost enzymes was comparable to that of the mammalian homologue (positive control lane).

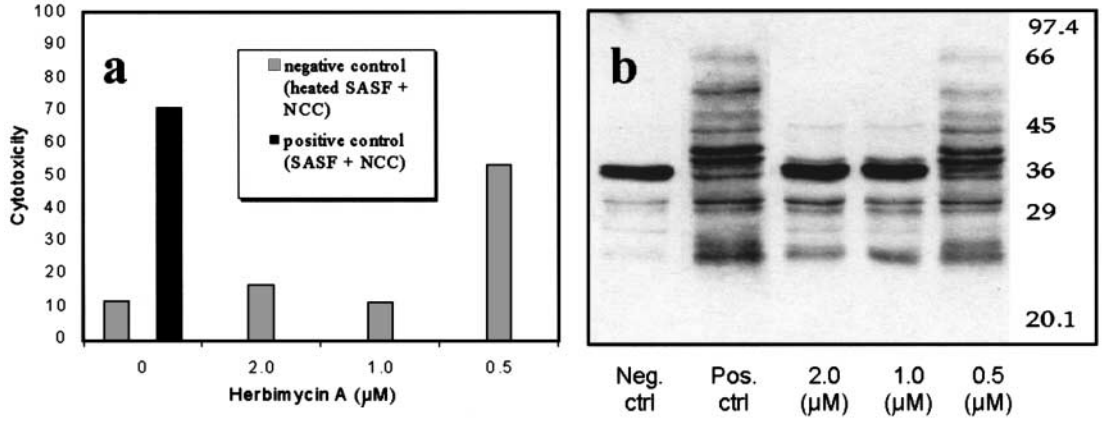

Fig. 4. HA inhibits the activation of NCC by SASF. Purified NCC from the peripheral blood of naïve tilapia were pre-incubated for 4 h with and without different concentrations of HA. Cells were washed and treated with SASF for 4 h. Cells treated with heat-inactivated SASF (65°C/15 min) were used as a negative control (ctrl). Finally, cells were washed and added to labeled HL-60 cells (E:T ratio 160:1). (a) Supernatants were harvested and radioactivity measured following 18 h cocultivation. (b) At the same time, the same cells were lysed and used for Western blots from 1-D gels, probed with anti-phosphotyrosine monoclonal antibodies

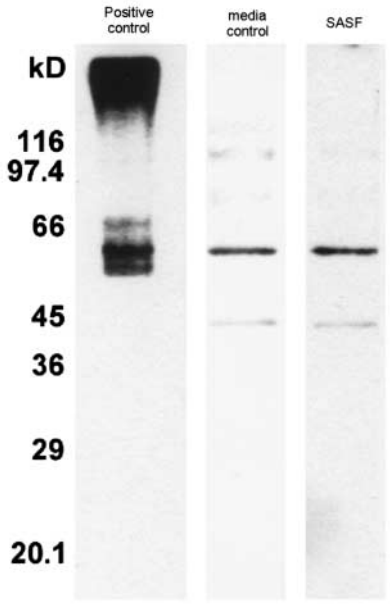

Fig. 5. *lck* is constitutively expressed in both naïve and activated NCC. Freshly isolated NCC (5×10^6) from peripheral blood of naïve tilapia were resuspended in RPMI followed by incubation in the presence and absence of SASF. NCC were then washed twice with cold PBS and lysed for 30 min in Triton-X100 lysis buffer. After centrifugation at 4°C for 15 min, supernatants were collected as cytoplasmic extracts. Samples were heated with 4× loading buffer, separated in a 11.5% acrylamide gel and transferred to membranes for probing with anti-*lck* polyclonal antibodies (1:2000). Lane A: positive control of mammalian *lck* provided with the antibody. Lane B: NCC treated with media alone; Lane C: NCC incubated in the presence of SASF

DISCUSSION

Although the importance of lymphokines in natural and adquired immune responses is without question, very few cytokines have been sequenced to date in lower vertebrates (Secombes et al. 1998). For this and other reasons (lack of phenotyping reagents), very little is known about the specific molecular mechanisms of activation of fish lymphocytes during the typical immune response to viral and bacterial infections, parasite infestations and stress conditions. Reversible protein phosphorylation is the predominant strategy used to control the metabolic activity of proteins in eukaryotic cells (Johnson & Barford 1993). Thus, phosphorylation and dephosphorylation reactions are essential in signal transduction events that control several cellular processes (Cohen 1989). Target cellular proteins are phosphorylated at specific serine, threonine or tyrosine residues by protein kinases and the phosphate group is then removed by the action of specific protein phosphatases (Alexander 1990). We have presented data showing evidence of downstream signaling events that occur in tilapia NCC following activation by cytokine-like factors. It is clear that interleukins can induce different cellular responses such as lymphocyte activation, growth and differentiation (Arai et al. 1990). A complex signaling system in which protein phosphorylation by nonreceptor PTKs appears to be critical (Arai et al. 1990, Taniguchi 1995) mediates such diverse responses. This is the first report showing how the addition and removal of phosphate groups from key tilapia NCC proteins affects effector functions. In particular, phosphorylation was shown to be indispensable in the activation of cytotoxic activity of tilapia NCC induced by serum cytokine-like factors.

The tyrosine phosphatase specific inhibitor sodium orthovanadate was shown to be an important immunomodulator of nonspecific cytotoxic cell activity in tilapia NCC. Similar results were reported with NCC isolated from channel catfish *Ictalurus punctatus* (Evans & Jaso-Friedmann 1994). In both cases, this immunomodulation was a signaling event occurring at the level of an individual NCC. In addition, sodium orthovanadate showed protective effects against camptothecin-induced apoptosis of NCC. Although the role of tyrosine phosphorylation in cell growth and differentiation has been widely studied, very little is known about the role of phosphorylation in the regulation of programmed cell death (Yang et al. 1996, Kuli et al. 1997, Ueno et al. 1997, Lifshitz et al. 1998). Apoptosis plays important physiological roles not only in immune cells but in many other tissues.

One model that may confirm the regulation of signal transduction responses by phosphatases on NCC are studies related to the mechanisms of how the leukocyte common antigen CD45 participates in NK and T cell activation processes. It has been demonstrated that in rat NK cells CD45 regulates the inhibitory signal pathway after self MHC class I recognition. This event occurs apparently by dephosphorylation of certain cellular proteins (Giezeman-Smits et al. 1999). Additionally, biochemical analyses and immunofluorescence studies have shown that the tyrosine kinase associated with CD45 in NK cells is $p56^{lck}$ and that cross-linking of CD45 results in the activation of $p56^{lck}$ (Xu & Chong 1995). Thus, CD45 may regulate $p56^{lck}$ kinase, which initiates the cascade of intracellular tyrosine phosphorylation events that lead to the transcription and secretion of cytokines such as IFN-gamma (Shen et al. 1995, Xu & Chong 1995).

CD45 also plays an important role in T cell activation mechanisms by binding $p56^{lck}$ (Sieh et al. 1993, Lee et al. 1996, D'Oro & Ashwell 1999, Gervais & Veillette 1997, Pingel et al. 1999) and $p59^{fyn}$ as substrates (Hurley et al. 1993, Imbert et al. 1996). It has also been demonstrated how the stimulation of the T-cell antigen receptor-CD3 complex signaling pathway by pervana-

date is mediated by inhibition of CD45 (Imbert et al. 1996). In addition, CD45-dependent events such as tyrosine phosphorylation of Shc (Ghosh & Miller 1995), major histocompatibility class II-mediated signal transduction (Greer et al. 1998), and the stimulation of the T-cell antigen receptor-CD3 (Imbert et al. 1996) complex signaling pathway also involve the activation of the tyrosine kinases *lck* and *fyn*. Although we do not know if CD45 is expressed in tilapia NCC, these data, combined with the fact that *lck* and *fyn* were found constitutively expressed on tilapia NCC, confirm our hypothesis that phosphatases inhibited by vanadate are directly associated with cytotoxicity.

The relationship between the inhibition of tyrosine kinase activity by HA and the inactivation of cytolysis in NK cells and CD3-large granular lymphocytes has been demonstrated in both natural cytotoxicity and antibody-dependent cellular cytotoxicity (O'Shea et al. 1992, Murakami et al 1998). The present report suggests that PTKs play an important role in the positive regulation of NCC activated by SASF. The inhibition occurred when NCC were treated with HA concentrations between 1.0 and 2.0 µM (Fig. 4).

From the data presented above, it appears that factors with cytokine bioactivity play an important role in coordinating NCC responses of fish as in other vertebrates. Those factors appear within minutes in the serum of stressed fish in response to external conditions such as cold water stress or infectious diseases. The activation of NCC cells by serum cytokines is phosphorylation-induced; in addition, the signal transduction events that regulate the NCC activity are probably associated with the *src* family of PTKs. Furthermore, activation of killing may be related to the protection of NCC from activation-induced program cell death that occurs following target cell contact (Bishop et al. 2000). Future studies will be directed towards the identification of other second messengers that control lytic processes and killing kinetics of NCC.

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